

Inhibitory activity of diarylamidine derivatives on murine leukemia L1210 cell growth

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Key words: diarylamidines, diarylimidazolines, murine leukemia L1210, DNA synthesis

Summary

A series of 96 diarylamidine (and diarylimidazoline) derivatives were evaluated for their inhibitory effects on the growth and DNA synthesis of murine leukemia L1210 cells. The amidino- and imidazolino-substituted aryl moieties of the compounds consisted of phenyl, indole, indene, benzofuran, benzo[b]thiophene or benzimidazole. Several of these compounds were found to inhibit L1210 cell proliferation with an ID₅₀ (50% inhibitory dose) of 1 µg/ml or lower. Structure-function analysis revealed that the antitumor cell activity of the diarylamidines depended on the planarity of the molecule, the presence of amidino- (or, preferably, imidazolino-) groups on both aryl moieties, the nature of the bridge connecting the two aryl moieties (preferably no bridge at all, phenoxy or ethene) and, finally, the nature of the aryl moieties (preferably, benzofuran or benzo[b]thiophene). Hence, compound 20 (6-(2-imidazolin-2-yl)-2-[4-(2-imidazolin-2-yl)phenyl] benzo[b]thiophene) emerged as the most potent inhibitor of L1210 cell growth (ID₅₀: 0.21 µg/ml). Its inhibitory potency was similar to that of the well-known trypanocidal drug ethidium bromide (compound 98). For all diarylamidine derivatives taken together, some correlation ($r = 0.612$) was noted between the log ID₅₀ for L1210 cell proliferation and the log ID₅₀ for L1210 cell DNA synthesis (as monitored by [*methyl*-³H]dThd incorporation). These findings suggest that the inhibitory effects of the diarylamidines on L1210 cell proliferation may at least partially reside in an inhibition of DNA synthesis. Compound 41 (2,2'-vinylenedi-1-benzofuran-5-carboxamidine), that exhibited a potent antitumor activity *in vitro* (ID₅₀: 1.5 µg/ml), was further evaluated for its antitumor efficacy *in vivo* and found to increase the median survival time of L1210 cell-inoculated BDF₁ mice up to 204%, if administered at a dose of 200 mg/kg.

Introduction

Aromatic diamidines such as stilbamidine and pentamidine inhibit the growth of protozoa, bacteria, fungi and tumor cells, generally at concentrations well below those found to be toxic for the host (1). Another diamidine derivative, DAPI (4',6-diamidino-2-phenyl-indole), has been shown to form fluorescent complexes with double-stranded DNA (2, 3) and can be used for the fluorescent staining of bacteria, spermatozoa, mycoplasmata (4) and

chromosomes (5). DAPI and its congeners strongly interact with A-T regions of DNA (6-8), thereby suppressing the DNA-directed RNA and DNA polymerases, and this inhibitory activity may obviously contribute to the growth-inhibitory effects that have been observed with diarylamidines in various cell systems. Several diarylamidines are potent inhibitors of reverse transcriptase (RNA-directed DNA polymerase) (9) and arginine-specific proteases (trypsin, thrombin, kallikrein) (10), and while there is no direct correlation between

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anti-DNA polymerase activity and anti-protease activity (9), both activities depend on rather specific structural requirements.

We have now evaluated a large series of diarylamidine (and diarylimidazoline) derivatives for their inhibitory effects on the growth of murine leukemia L1210 cells. Because of their strong affinity for DNA, the compounds were also evaluated for their inhibitory effects on cellular DNA synthesis (monitored by [*methyl*-³H]dThd incorporation). These studies were aimed (i) at delineating the structural features that determine the cell growth-inhibiting activity of diarylamidine derivatives, (ii) at gaining further insight in their mechanism of cytotoxic action, and, hopefully, (iii) at detecting some compounds with potential anticancer activity.

Based on the structure-function analysis one should be able to establish the guidelines for the synthesis of new and more potent inhibitors of tumor cell proliferation.

Materials and methods

Cells. Murine leukemia L1210 cells were grown in 75 cm² tissue culture flasks (Falcon 3024F; Becton Dickinson France S.A., Grenoble, France) in Eagle's minimal essential medium, supplemented with 10% (v/v) inactivated fetal calf serum (Gibco Bio-Cult, Glasgow, Scotland), 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland), 0.075% (w/v) NaHCO₃ (Flow Laboratories) and 25 units/ml of nystatine (S.A. Labaz N.V., Brussels, Belgium). The L1210 cell line was found to be *Mycoplasma*-free.

Chemicals. The diarylamidine derivatives listed in Tables 1 through 6 were synthesized as described previously. The references for the synthesis of the compounds are as follows: 1–4, 9, 26 (ref. 11); 11, 19, 21–23, 75–77, 83, 88, 95 (ref. 12); 14–17, 24, 25 (ref. 13); 30–34, 49, 65 (ref. 14); 39–41, 44–46, 52, 55–57, 96 (ref. 15); 10, 12, 13, 42, 43, 47, 48, 50, 51, 78–82, 84–87 (ref. 16); 10, 20, 36, 37, 50, 59, 60, 66–68, 70–73, 94 (ref. 17); 12, 13, 47, 48, 51, 79–82, 84, 85, 87 (ref. 18); 42, 43, 53,

54, 78, 86 (ref. 19); 64 (ref. 20); 35, 38 (ref. 21); 6 (ref. 22); 7 (ref. 23); 58 (ref. 24); 63 (ref. 25); 89 (ref. 26); 8, 18, 44, 92 (ref. 27); 61, 62, 69, 74, 90, 91, 93 (ref. 28).

Reference compounds. Ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide) was obtained from Calbiochem (Los Angeles, California, U.S.A.), whereas tilorone dihydrochloride [2,7-bis[2-(diethylamino)ethoxy]-fluoren-9-onedihydrochloride] was supplied by Richardson-Merrell Inc. (Cincinnati, Ohio, U.S.A.). Berenil (diminazene diacetate) was a product of Hoechst A.G. (Frankfurt/Mainz, F.R.G.), whereas stilbamidine isethionate (4,4'-stilbenedicarboxamidine diisethionate) and pentamidine isethionate (4,4'-diamidino- α,ω -diphenoxypentane isethionate) were products of May and Baker Ltd (Dagenham, England).

Radiochemicals. The radiolabelled nucleoside [*methyl*-³H]dThd (specific radioactivity, 38 Ci/mmole) was obtained from the Institute of Radio Elements (IRE, Fleurus, Belgium).

Inhibition of tumor cell growth. All assays were performed in Linbro microplates (model FB-48-TC, Linbro Chemical Company, New Haven, Connecticut, U.S.A.). To each well were added 5×10^4 L1210 cells and a given amount of the test compound. The cells were allowed to proliferate for 48 h at 37°C in a humidified, CO₂-controlled atmosphere. The growth of the cells was linear during this 48 h-incubation period. At the end of the incubation period, the cells were counted in a Coulter counter (Coulter Electronics Ltd, Harpenden Herts., England). The ID₅₀ (50% inhibitory dose) was defined as the concentration of compound that reduced the number of living cells by 50%.

Inhibition of [*methyl*-³H]dThd incorporation. The incorporation of [*methyl*-³H]dThd into cellular DNA was also measured in Linbro microplates. To each well were added 10^5 L1210 cells, 6.5 pmoles (0.25 μ Ci) of [*methyl*-³H]dThd, and a given amount of the test compound. The cells were al-

lowed to proliferate in humidified, CO₂-controlled atmosphere. At the end of this incubation period, the cells were harvested into wells (200 μ l) by using a multiwell harvester (type A, Ann Arbor, MI). The filters were washed with phosphate-buffered saline (pH 7.4) and then with 100% ethanol. The filters were then dried and counted in a toluene-based scintillation fluid.

In vivo tests. ICR (Inbred Charles River) and DBA/2 mice were used in the *in vivo* test. The mice were anesthetized and inoculated with 0.1 ml of a suspension of L1210 cells per cell. The cells were cultured in a medium span (MLS) thallium (25 days) if cells were used. The peritoneal cavity was washed with 0.2 ml of saline solution.

Results

According to the results of the *in vivo* tests, the compounds can be regarded as being active in which the amino groups are linked to the carbon-oxygen atom. Compounds C and D were found to be the most active. The benzo[b]thiophene (Table 1, 3 and 4) compounds were also active. The zolono-substituted compounds were also active.

lowed to proliferate for 20 h at 37°C in a humidified, CO₂-controlled atmosphere. At the end of this incubation period, the contents of the wells (200 µl) were brought onto 25-mm glass fiber filters (type A/E, Gelman Instrument Company, Ann Arbor, Michigan, U.S.A.), mounted on a Millipore 3025 sampling manifold apparatus. The filters were washed twice with cold PBS (phosphate-buffered saline), twice with cold 10% trichloroacetic acid, twice with cold 5% trichloroacetic acid, once with cold ethanol, and once with cold ether. The filters were then allowed to dry for 10 min at 60°C and assayed for radioactivity in a toluene-based scintillant.

In vivo tests. Five- to six-week-old BDF₁ (C₅₇BL × DBA/2) mice weighing 18 to 20 g were used for the *in vivo* tests. They were housed at 8 per cage and inoculated intraperitoneally with 5 × 10⁶ L1210 cells per 0.2 ml PBS per mouse. The L1210 cells were cultured *in vitro* before they were injected into mice. As a consequence, the median life span (MLS) that has been observed in these experiments (25 days) is longer than it should be (7 days) if cells were used that have been passaged in the peritoneal cavity of BDF₁ mice. The test compounds were also injected intraperitoneally, one day after L1210 cell inoculation. Control mice received 0.2 ml of a PBS solution.

Results

According to the type of the amidino-substituted skeleton, the compounds were divided into six classes (Tables 1–6). Class D (Table 4) can actually be regarded as an extension of class A (Table 1) in which the amidino- (or imidazolino-) substituted rings are linked by a carbon (carbon-nitrogen or carbon-oxygen) chain of varying length. Classes A, C and D were further subdivided according to the type of the heterocyclic ring (indole, benzofuran, benzo[b]thiophene, indene or benzimidazole) (Table 1, 3 and 4). Class B (Table 2) contains those compounds where each of the amidino- (or imidazolino-) substituted rings is a benzene. Class E is composed of a few compounds with the aryl moi-

ties (indoles) linked together through a C₂–C₆ linkage, instead of the usual C₂–C₂ linkage (class C compounds, Table 3). Some miscellaneous compounds were brought together in Table 6. All compounds tested are listed in Tables 1–6 with their respective ID₅₀ (50% inhibitory dose) values for L1210 cell growth and DNA synthesis [*methyl*-³H]dThd incorporation).

Inhibitory effect on L1210 cell growth

Within class A compounds (Table 1), the highest inhibitory activity on L1210 cell growth (ID₅₀: 0.21 µg/ml) was noted for compound 20, which was about 20 times more active than the standard diarylamidine (DAPI, compound 4). This increase in activity was probably due to substitution of amidino by imidazolino groups and not to substitution of the ring nitrogen by sulfur. Indeed, within the subclass of the benzo[b]thiophene analogues substitution of imidazolino for amidino groups resulted in a significant increase in antitumor cell activity (compare compounds 19 with 15 and 20 with 17). Also substitution of the amidino by guanidino or CH = NNHC(=NH)NH₂ groups caused a marked increase in antitumor cell activity (compare compounds 12 with 9, and 22 and 23 with 17). For the amidino-substituted indoles, the degree of antitumor cell activity depended on the position of the amidino groups, thus in order of decreasing activity: 6,4' > 5,4' > 6,3' > 5,3' (4 > 2 > 3 > 1). For the amidino-substituted benzo[b]thiophenes, the position of the amidino groups did not significantly alter the antitumor cell activity (compounds 14 through 18). Introduction of an amino group in position 3 decreased the activity (compare compounds 5 with 4, and 18 with 15). Molecular planarity seems to be equally important for inhibitory activity. If the planar state of compound 15 was reduced by a 1,1-dioxy-substitution (compounds 24 and 25) and hydrogenation of carbons 2 and 3 (compound 25), there was a concomitant reduction in inhibitory activity. In general, isosteric replacements, i.e. NH by CH₂ (compounds 4 and 26) or NH by S (compounds 4 and 17), did not significantly alter the antitumor cell activity. However, for the 5,3' diamidines, replacement of NH by S (compounds 1 and 14) en-

Table 1. Class A compounds.

Am = —C(=NH)NH_2 Im = —C(=N)NCH_2

Compound no.	X	Y	R ₁	R ₂	ID ₅₀ ^c (μg/ml)	
					L1210 cell growth	[methyl- ³ H]dThd incorporation
I. Indoles						
1	NH	CH	Am ^a (5)	Am (3)	27.0	> 100
2	NH	CH	Am (5)	Am (4)	8.90	> 100
3	NH	CH	Am (6)	Am (3)	15.4	> 100
4	NH	CH	Am (6)	Am (4)	4.15	> 100
5	NH	CNH ₂	Am (6)	Am (4)	> 100	> 100
6	NH		Am (6)	Am (4)	> 19.4	> 100
7	NH	CH	Am (6)	OCH ₃ (3) OCH ₃ (4) OCH ₃ (5)	47.1	31.4
8	NH	CH	Im ^b (6)	Im (4)	2.82	58
II. Benzofurans						
9	O	CH	Am (5)	Am (4)	7.65	> 100
10	O	CH	Im (5)	Im (4)	1.52	14.1
11	O	CH	Am (5)	NH ₂ (4)	2.19	26.5
12	O	CH	NHC(=NH)NH ₂ (5)	NHC(=NH)NH ₂ (4)	0.620	31.5
13	O	CH	N=CHN(CH ₃) ₂ (5)	N=CHN(CH ₃) ₂ (4)	2.30	> 100
III. Benzo[b]thiophenes						
14	S	CH	Am (5)	Am (3)	6.93	> 100
15	S	CH	Am (5)	Am (4)	6.35	> 100
16	S	CH	Am (6)	Am (3)	8.43	> 100
17	S	CH	Am (6)	Am (4)	7.95	> 100
18	S	CNH ₂	Am (5)	Am (4)	14.5	> 100
19	S	CH	Im (5)	Im (4)	0.492	6.3
20	S	CH	Im (6)	Im (4)	0.210	5.0
21	S	CH	NHC(=NH)NH ₂ (5)	NHC(=NH)NH ₂ (4)	0.560	> 100
22	S	CH	NHC(=NH)NH ₂ (6)	NHC(=NH)NH ₂ (4)	0.980	—
23	S	CH	CH=NNHC(=NH)NH ₂ (6)	CH=NNHC(=NH)NH ₂ (4)	0.590	27.5
24	SO ₂	CH	Am (5)	Am (4)	93.0	> 100
25	SO ₂	CH ₂	Am (5)	Am (4)	> 100	> 100
IV. Indene						
26	CH ₂	CH	Am (6)	Am (4)	6.55	> 100

^a For structure of amidino (Am), see structure over column heads.^b For structure of imidazolino (Im), see structure over column heads.^c ID₅₀ = inhibitory dose-50.^d DAPI.

All experiments were done in duplicate or triplicate.

Table 2. Class B compounds.

Compound no.

27^d28^e29^f

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^{a-c} See corresponding^d 27: stilbamidine is^e 28: berenil.^f 29: pentamidine is

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



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Table 2. Class B compounds.

$$R_1 - \text{C}_6\text{H}_4 - Z - \text{C}_6\text{H}_4 - R_2$$

Compound no.	Z	R ₁	R ₂	ID ₅₀ ^c (μg/ml)	
				L1210 cell growth	[methyl- ³ H]dThd incorporation
27 ^d	CH=CH	Am ^a	Am	22.6	> 100
28 ^e	NH-N=N	Am	Am	14.6	> 100
29 ^f	O(CH ₂) ₅ O	Am	Am	0.745	29.5
30		Am	Am	5.00	69.0
31		Am	Am	17.8	> 100
32		Am	Am	7.70	91
33		Am	Am	36.7	> 100
34		Am	Am	5.50	> 100
35	p-NHCO-C ₆ H ₄ -CONH	Am	Am	> 100	> 100
36	NH-N=N	Im ^b	Am	17.5	> 100
37	NH-N=N	Im	Im	15.8	66.3
38	p-NHCO-C ₆ H ₄ -CONH	Im	Im	3.14	> 100

^{a,c} See corresponding footnotes to Table 1.^d 27: stilbamidine isethionate.^e 28: berenil.^f 29: pentamidine isethionate.

hanced the activity fourfold.

Within class B compounds (Table 2), the trypanocidal drug pentamidine isethionate (compound 29) displayed the greatest inhibitory effect on L1210 cell proliferation (ID₅₀: 0.74 μg/ml). Two other trypanocidal drugs, berenil (28) and stilbamidine isethionate (27) were 20–30 times less inhibitory. When the bridge connecting the two benzene rings contained a NH-N=N group, substitution of the amidino by one or two imidazolino groups had no significant influence on the antitumor cell activity (compare compound 28 with compounds 36 and 37). However, when the bridge between the two benzenes consisted of p-NHCO-C₆H₄-CONH, imidazolino substituents conferred a much higher activity than did the amidino groups. Heterocyclic bridges such as pyrrole (compound 30), furan (compound 32) or thiophene (compound 34) gave similar inhibitory activities. In contrast, an imidazole (com-

pound 31) or oxadiazole bridge (compound 33) led to a decreased antitumor cell activity.

Within class C compounds (Table 3), three compounds (40, 48 and 50) inhibited L1210 cell proliferation at an ID₅₀ of less than 1 μg/ml. As noted above for the class A compounds, substitution of the amidino by imidazolino groups brought about a (slight) increase in antitumor cell activity (compare compounds 50 with 41, and 51 with 47). The positioning of the amidino groups did not drastically influence the inhibitory effects on tumor cell growth. If the amidino groups of both benzofuran rings were moved from position 5 (compound 41) to position 6 (compound 43), there was a threefold decrease in inhibitory activity. However, if only one of the amidino groups was moved from position 5 (compound 41) to position 6 (compound 42), no substantial difference in inhibitory activity was observed. This was also the case for the positional isomers of

Table 3. Class C compounds.

Table 3. Class C compounds

Compound no.	X ₁	Y	X ₂	Z	R ₁	R ₂	ID ₅₀ ^c (μg/ml)	
							L1210 cell growth	[methyl- ³ H]dThd incorporation
I. Benzofuran/benzofuran								
39	O	CH	O	CH ₂	Am ^a (5)	Am (5)	4.00	> 100
40	O	CH	O	(CH ₂) ₂	Am (5)	Am (5)	0.96	58.7
41	O	CH	O	CH=CH	Am (5)	Am (5)	1.51	> 100
42	O	CH	O	CH=CH	Am (5)	Am (6)	1.66	54.8
43	O	CH	O	CH=CH	Am (6)	Am (6)	4.55	71
44	O	CCH ₃	O	CH=CH	Am (5)	Am (5)	3.09	48.5
45	O	CH	O	CH=CCH ₃	Am (5)	Am (5)	13.6	> 100
46	O	CH	O	CH ₂ CHCH ₃	Am (5)	Am (5)	1.06	> 100
47	O	CH	O	(CH=CH) ₂	Am (5)	Am (5)	2.54	> 100
48	O	CH	O	(CH=CH) ₃	Am (5)	Am (5)	0.636	20.9
49	O	CH	O		Am (5)	Am (5)	4.78	> 100
50	O	CH	O	CH=CH	Im ^b (5)	Im (5)	0.497	2.65
51	O	CH	O	(CH=CH) ₂	Im (5)	Im (5)	1.55	6.80
II. Benzofuran/indole								
52	O	CH	NH	—	Am (5)	Am (5)	2.92	> 100
53	O	CH	NH	CH=CH	Am (5)	Am (5)	1.12	36.0
54	O	CH	NH	CH=CH	Am (5)	Am (6)	1.51	65.2
III. Benzofuran/benzo[b]thiophene								
55	O	CH	S	CH=CH	Am (5)	Am (5)	1.41	> 100
IV. Benzo[b]thiophene/benzo[b]thiophene								
56	S	CH	S	CH=CH	Am (5)	Am (5)	6.60	> 100
V. Benzimidazole/benzimidazole								
57	NH	N	NH	CH=CH	Am (5)	Am (5)	15.2	52.1
58	NH	N	NH	NH	Am (5)	—	18.0	22.4
VI. Indole/indole								
59	NH	CH	NH	C ₆ H ₄ -O-C ₆ H ₄	Am (6)	Am (6)	5.31	72
60	NH	CH	NH	C ₆ H ₄ -O-C ₆ H ₄	Im (6)	Im (6)	> 100	> 100
61	NH	CH	NH	—	Im (6)	Im (6)	> 100	> 100
62	NH	CH	NH	—	Am (6)	Am (6)	3.53	> 100
VII. Benzo[b]thiophene/indole								
63	S	CH	NH	CH=CH	Im (6)	Im (6)	11.4	82.3

^{a-c} See corresponding footnotes to Table 1.

indole (compounds 53 and 54). The inhibitory activity of the bis(amidinobenzofuran) and bis(imidazolinobenzofuran) derivatives was influenced by the nature of the bridge connecting the two amidino- (or imidazolino-) substituted rings: most effective were the compounds with an ethane (compound 40) or ethene bridge (compounds 41, 42, 50, 53–55). A methane bridge (compound 39) gave a fourfold

lower activity than an ethane bridge (compound 40), whereas an isopropane bridge (compound 46) gave about the same activity. However, a substantial decrease in activity was noted upon introduction of an isopropene bridge (compound 45). Lengthening of the connecting ethenyl chain by one ethenyl unit did not cause marked changes in antitumor cell activity of the bis(amidinobenzofuran) derivatives

Table 4. Class I

Compound no.

I. Indoles

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II. Benzofurans

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83
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85
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87

III. Benzo[b]thio

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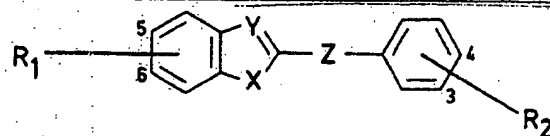
IV. Benzimidazo

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Table 4. Class-D compounds.



Compound no.	X	Y	Z	R ₁	R ₂	ID ₅₀ ^c (μg/ml)	
						L1210 cell growth	[methyl- ³ H]dThd incorporation
I. Indoles							
64	NH	CH	CH=CH	Am ^a (6)	Am (4)	3.60	± 100
65	NH	CH	C ₆ H ₄ -O	Am (6)	Am (4)	1.30	14.8
66	NH	CH	C ₆ H ₄ -O	Am (6)	Am (3)	0.53	38.6
67	NH	CH	C ₆ H ₄ -O	Am (5)	Am (4)	1.97	15.3
68	NH	CH	CH=CH-C ₆ H ₄ -O	Am (6)	Am (4)	12.4	62.8
69	NH	CH	mC ₆ H ₄ -O	Am (6)	Am (4)	2.20	34.5
70	NH	CH	C ₆ H ₄ -O	Im ^b (6)	Im (4)	0.50	13.3
71	NH	CH	C ₆ H ₄ -O	Im (6)	Im (3)	1.49	21.5
72	NH	CH	C ₆ H ₄ -O	Im (5)	Im (4)	2.17	> 100
73	NH	CH	CH=CH-C ₆ H ₄ -O	Im (6)	Im (4)	1.28	23.8
74	NH	CH	mC ₆ H ₄ -O	Im (6)	Im (4)	3.23	> 45.3
II. Benzofurans							
75	O	CH	CH=CH	Am (5)	Am (4)	7.30	60
76	O	CH	(CH ₂) ₂	Am (5)	Am (4)	1.48	> 100
77	O	CH	CH=CCH ₃	Am (5)	Am (4)	0.651	> 100
78	O	CH	CONH	Am (5)	Am (4)	3.75	> 100
79	O	CH	CH=NNH	Am (5)	Am (4)	3.55	> 100
80	O	CH	(CH=CH) ₂	Am (5)	Am (4)	1.41	59.5
81	O	CH	(CH=CH) ₃	Am (5)	Am (4)	1.33	12.0
82	O	CH	CH=CH-C ₆ H ₄ -O	Am (5)	Am (4)	1.88	22.0
83	O	CH	C ₆ H ₄ -N=NNH	Am (5)	Am (4)	12.0	68.6
84	O	CH	CH=CH	Im (5)	Im (4)	0.550	5.55
85	O	CH	(CH=CH) ₂	Im (5)	Im (4)	1.21	4.30
86	O	CH	CONH	Am (5)	Im (4)	11.7	> 100
87	O	CH	CH=CH-C ₆ H ₄ -O	Im (5)	Im (4)	0.450	2.17
III. Benzo[b]thiophene							
88	S	CH	CONH	Am (5)	Am (4)	1.48	> 100
IV. Benzimidazole							
89	NH	N	NH	Am (5)	Am (4)	27.8	> 100

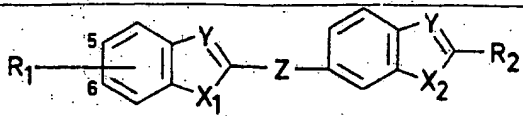
^{a-c} See corresponding footnotes to Table I.

(compare compound 41 with compound 47), although it decreased the activity of the bis(imidazolinobenzofuran) derivative by threefold (compare compound 50 with 51). Lengthening of the connecting ethenyl chain by two ethenyl units caused a fourfold increase in the inhibitory activity of the bis(amidinobenzofuran) derivative (com-

pare compound 47 with 48). In some cases, isosteric replacements caused major changes in inhibitory activity. For example, substitution of sulfur for oxygen in both benzofuran rings resulted in a four- to fivefold decrease of activity (compounds 41 and 56). Modification of the benzofuran rings to benzimidazole rings led to a 10-fold

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Table 5. Class E compounds.

								
Compound no.	X ₁	Y	X ₂	Z	R ₁	R ₂	ID ₅₀ ^c (μg/ml)	
							L1210 cell growth	[methyl- ³ H]dThd incorporation
<i>Indole/indole</i>								
90	NH	CH	NH	CH=CH	Im ^b (6)	Im	24.0	> 100
91	NH	CH	NH	—	Im (6)	Im	13.3	> 100

^{b, c} See corresponding footnotes to Table 1.

decrease of activity (compounds 41 and 57). However, replacement of one benzofuran ring by indole (compound 53) or benzo[b]thiophene (compound 55) did not markedly change the antitumor cell activity. The activity of the bis(indole) derivatives was critically influenced by the nature of the substituents: in contrast to the observations made for the class A and class B compounds, substitution of imidazolino for amidino groups resulted in a complete loss of inhibitory activity (compare compounds 59 with 60, and 61 with 62).

Within class D compounds (Table 4), maximal inhibitory activity as observed for compounds 66, 70, 84 and 87 (ID₅₀: ~ 0.5 μg/ml). While substitution of both amidino by imidazolino groups caused little, if any, change in inhibitory activity for compounds 65–67 and 69 (compare with compounds 70–72 and 74), such substitution led to a ten-fold increase in activity for compound 68 (compare with compound 73). Among the benzofurans, the imidazolino-substituted derivatives proved, in general, more effective than the corresponding amidino-substituted derivatives (compare compounds 87 with 82, and 84 with 75). As demonstrated by the comparable activity of compounds 65–67, on the one hand, and compounds 70–72, on the other hand, the positioning of the amidino or imidazolino groups had no major impact on the cell growth-inhibiting properties of the indoles. In contrast to the class C compounds where replacement of an ethane or ethenyl by an isopropene bridge led to a considerable decrease of activity, an

isopropene bridge appeared compatible with significant antitumor cell activity for class D compounds (compare compound 77 with compounds 75 and 76). Also, lengthening of the connecting ethenyl chain by one or two ethenyl units resulted in a marked increase of activity (compare compound 75 with compounds 80 and 81).

Of class E only two compounds (90 and 91) (Table 5) were tested. None showed a considerable antitumor cell activity.

Among the miscellaneous compounds (Table 6), the trypanocidal drug ethidium bromide (98) showed the highest antitumor cell activity (ID₅₀: 0.22 μg/ml). Compounds 97 (tilorone dihydrochloride) and 92 were also endowed with a marked inhibitory activity. Other compounds, such as the bis-amidino-substituted indoles (93 and 94), proved virtually inactive.

Inhibitory effect on L1210 cell DNA synthesis

Of class A compounds (Table 1), the most potent inhibitors of L1210 cell growth, compounds 20 and 19, showed also the strongest inhibitory effect on L1210 cell DNA synthesis. However, compound 21, which inhibited tumor cell growth at almost the same concentration as compound 19, did not prove effective as inhibitor of cellular DNA synthesis. In fact, most of the compounds for which the ID₅₀ for L1210 cell proliferation fell within the 2–20 μg/ml range failed to inhibit [methyl-³H]dThd incorporation at a concentration of 100 μg/ml. Despite the

Table 6. Miscellaneous

Compound no.

92

93

94

95

96

97^d98^e^c See corresponding^d 97: tilorone dihy^e 98: ethidium bro

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Table 6. Miscellaneous compounds.

Compound no.	Structure	ID ₅₀ ^c (μg/ml)	
		L1210 cell growth	[methyl- ³ H]dThd incorporation
92		3.93	15.3
93		71.3	> 100
94		60.5	> 100
95		32.2	> 100
96		8.45	> 100
97 ^d		3.15	14.6
98 ^e		0.22	8.0

^c See corresponding footnote to Table 1.^d 97: tilorone dihydrochloride.^e 98: ethidium bromide.

failure of several class A compounds to inhibit cellular DNA synthesis, their log ID₅₀ for L1210 cell proliferation and log ID₅₀ for [methyl-³H]dThd incorporation showed a relatively close correlation ($r = 0.569$).*

Among class B compounds (Table 2), the most potent tumor cell growth inhibitor, pentamidine isethionate (29), inhibited [methyl-³H]dThd incorporation only at a 40-fold higher concentration than L1210 cell proliferation. For only four compounds a correlation coefficient could be calculated and this amounted to 0.864.

For class C compounds (Table 3), the correlation between the log ID₅₀ for tumor cell growth

and the log ID₅₀ for cellular DNA synthesis was lower than for the class A compounds ($r = 0.465$). Compound 50, which was the most potent cell growth inhibitor, also exerted the strongest inhibitory effect on [methyl-³H]dThd incorporation, although it proved about 5-fold more potent in inhibiting cell growth than DNA synthesis. Various other compounds (i.e. 41, 46, 47, 55) which inhibited tumor cell proliferation at an ID₅₀ comprised between 1–2.5 μg/ml, did not affect [methyl-³H]dThd incorporation at a concentration of 100 μg/ml.

For class D compounds (Table 4), the correlation between log ID₅₀ for L1210 cell proliferation

* When determining the correlation coefficients, compounds with ID₅₀ values > 100 μg/ml were not taken into account.

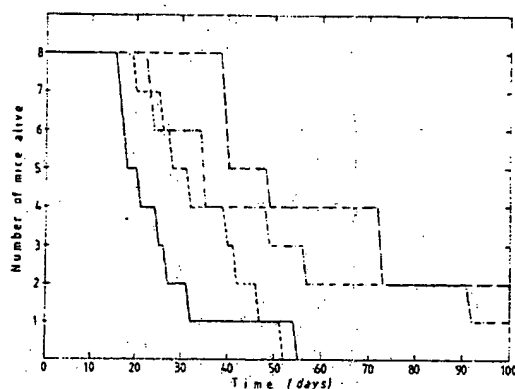


Fig. 1. Influence of compound 41 (diacetate salt) on the survival of BDF₁ mice inoculated intraperitoneally with 5×10^6 L1210 cells per mouse. Compound 41 was injected intraperitoneally as a single dose of either 200 mg/kg (—), 40 mg/kg (-.-.-), 8 mg/kg (.....) or 0 mg/kg (control) (—), one day after tumor cell inoculation.

and log ID₅₀ for [methyl-³H]dThd incorporation was higher than for the class A and C compounds ($r = 0.706$).

Among class E and the miscellaneous compounds (Tables 5 & 6), ethidium bromide (compound 98) turned out to be the most potent inhibitor of L1210 cell DNA synthesis. However, its ID₅₀ value for [methyl-³H]dThd incorporation was still 40 times higher than its ID₅₀ value for L1210 cell proliferation, and differed not much from the ID₅₀ values for compound 97 (tilorone dihydrochloride) and compound 92, two compounds which were about 10 times less potent as L1210 cell growth inhibitors than ethidium bromide. Taking together all compounds belonging to either class A, B, C, D or E, we found a correlation coefficient of 0.612.

Antitumor activity in vivo

Since compound 41 ranked among those diarylamidine derivatives that showed potent antitumor cell activity *in vitro* (ID₅₀: 1.51 μ g/ml), it was further evaluated for its antitumor activity *in vivo*. BDF₁ mice were injected intraperitoneally with 5×10^6 L1210 cells per mouse. The median life span of these mice was 25.0 days. Compound 41 was injected intraperitoneally as a single dose of 200

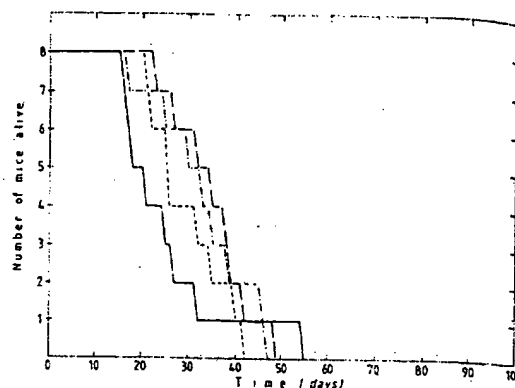


Fig. 2. Influence of ethidium bromide (98) on the survival of BDF₁ mice inoculated intraperitoneally with 5×10^6 L1210 cells per mouse. Ethidium bromide was injected intraperitoneally as a single dose of either 20 mg/kg (—), 4 mg/kg (-.-.-), 0.8 mg/kg (.....) or 0 mg/kg (control) (—), one day after tumor cell inoculation.

mg/kg, 40 mg/kg or 8 mg/kg, one day after tumor cell inoculation. As reference compound served ethidium bromide (98) which was administered at 20 mg/kg, 4 mg/kg or 0.8 mg/kg. Because of toxicity higher doses of this compound could not be administered.

The antitumor effects obtained with compounds 41 and 98 are presented in Figs. 1 and 2. Compound 41 proved quite efficient in prolonging the life span of tumor cell-inoculated mice. At a dose of 200 mg/kg it increased the median survival time up to 51 days (or 204% of control) and 2 out of 8 mice survived even at 100 days after tumor cell inoculation. At a 5-fold lower dose, compound 41 achieved a median survival time of 175%, and at 8 mg/kg, it increased the life span to 139%. Ethidium bromide (98) proved inferior to compound 41 in increasing the life span of L1210 cell-inoculated BDF₁ mice. When injected at the highest tolerated dose (20 mg/kg), compound 98 demonstrated an increase of the median survival time to 137%. At lower doses (4 mg/kg and 0.8 mg/kg) compound 98 extended the median life span to 132% and 118%, respectively.

Discussion

For a series of (zoline) deriva according to the strongest inhibi proliferation (2-yl)-2-[4-(2-ir phenyl)-2-am imidine isethio with 5,5'-bis 1-benzofuran

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Discussion

For a series of 96 diarylamidine (and diarylimidazoline) derivatives, divided into five classes (A-E) according to the nature of the aromatic rings, the strongest inhibitory effects on murine L1210 cell proliferation were observed with 6-(2-imidazolin-2-yl)-2-[4-(2-imidazolin-2-yl)phenyl] benzo[b]thiophene (20) among class A compounds, with pentamidine isethionate (29) among class B compounds, with 5,5'-bis-(2-imidazolin-2-yl)-2,2'-vinylene-1-benzofuran (50) among class C compounds, with 5-(2-imidazolin-2-yl)-1-(4-[4-(2-imidazolin-2-yl)phenoxy]-styryl)-1-benzofuran (87) among class D compounds and with 2-(2-imidazolin-2-yl)-6-[6-(2-imidazolin-2-yl)-2-indolyl]indole (91) among class E compounds. The cell growth-inhibiting potency of these compounds (with the exception of 91) corresponded to that of the known cytotoxic compound, ethidium bromide (98) (ID_{50} ranging between 0.2 and 0.75 $\mu\text{g/ml}$).

Although the doses required to inhibit L1210 cell growth were considerably lower than the ID_{50} values for [*methyl*- ^3H]dThd incorporation, the antitumor cell activity of the diarylamidines and diarylimidazolines may at least partially be ascribed to their suppressive effects on cellular DNA synthesis. Indeed, the log ID_{50} of the test compounds for L1210 cell growth and the log ID_{50} for [*methyl*- ^3H]dThd incorporation showed a reasonable correlation: $r = 0.612$ for all compounds together. Thus, the cytotoxic properties of the diarylamidine derivatives may be due to an impairment of DNA replication, which, in turn, may result from binding of the diamidines to A-T base pairs in DNA.

Previous studies have been focussed on the antiproteolytic and anticoagulant activities (10), anti-oncogenic DNA polymerase activity (9) and antifungal and antibacterial activities (29) of diarylamidine derivatives. The studies of Tidwell *et al.* (10) indicated that the antiproteolytic activity of diarylamidines did not correlate with their anticoagulant activity and that even for the antiprotease activity marked differences were noted, depending on the nature of the protease tested (trypsin, thrombin or kallikrein). A comparison of

structure-activity data with those previously reported by Tidwell *et al.* reveals that there is no direct correlation between anti-protease activity and anti-L1210 cell proliferation activity. For example, compound 10 was inactive as a trypsin inhibitor, yet fairly active as an L1210 cell growth-inhibitor. Whereas substitution of imidazolino for amidino generally increased the L1210 cell-growth inhibiting activity (e.g. compare compound 9 with 10, 15 with 19, 17 with 20, etc.), it annihilated the anti-protease activity (compare compound 14 with 17, and compound 24 with 32 in ref. 10). Furthermore, some very effective trypsin inhibitors (compounds 10 and 29 in ref. 10) were inactive as inhibitors of L1210 cell proliferation (compounds 5 and 25 in Table 1).

Similarly, no direct correlation could be established between the inhibitory effects of the diarylamidine derivatives on oncornaviral DNA polymerase (9) and their L1210 cell-inhibiting properties. For example, compound 27 in ref. 9, which proved barely active against oncornaviral DNA polymerase, exhibited the greatest L1210 cell-growth inhibiting activity among the class B compounds (compound 29 in Table 2). Also, compound 21, one of the most potent inhibitors of L1210 cell proliferation, was only weakly active as inhibitor of the oncornaviral DNA polymerase activity (compound 19 in ref. 9). Compound 83, an efficient inhibitor of oncornaviral DNA polymerase (compound 69 in ref. 9), showed only moderate activity as an inhibitor of L1210 cell proliferation.

While Anné *et al.* (29) observed similarities in the structural requirements for the antibacterial and antifungal properties of the diarylamidine derivatives, these structural similarities do not extend to the antitumor cell activity of the diarylamidines. For example, substitution of amidino by imidazolino groups diminished the antifungal activity (compare compounds 60 with 56, 59 with 57, 71 with 62, and 72 with 67 in ref. 29), but enhanced the L1210 cell growth-inhibiting activity (compare compounds 19 with 15, 20 with 17, 38 with 35, 70 with 65, and 84 with 75). Compounds 4 and 27, which proved to be the most potent antifungal compounds among class A and class B compounds

(compound 4 and 25 in ref. 29), respectively, were only weakly active as inhibitors of L1210 cell proliferation.

Thus, the structure-function relationship data obtained in this area and previous studies (9, 10, 29) point to important differences in the structural requirements of diarylamidine derivatives as either protease inhibitors or DNA polymerase inhibitors, or antifungal, antibacterial, or antitumor agents. The antitumor activity that has been demonstrated with compound 41 *in vivo* (BDF₁ mice inoculated with L1210 cells) suggested that further antitumor studies should be undertaken with those compounds that show the greatest potency as inhibitors of L1210 cell growth *in vitro*. These antitumor studies should also be extended to animal models other than the L1210 system.

Acknowledgements

We are indebted to Lizette van Berckelaer for excellent technical assistance and to Christiane Callebaut for skilled editorial help.

This study was supported by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Krediet no. 30048.75) and Geconcentreerde Onderzoeksacties (Conventie no. 76/81-IV), and from the Wilhelm-Sander-Stiftung.

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